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14. ABSTRACT
An amino acid hydrolysis and analysis system is requested from the Air Force Defense Research Science Program. The proposed instrumentation will further the current research effort funded by AFOSR , namely the "Site Directed Nucleation and Growth of Ceramic Films on Metallic Surfaces" by allowing for the qualitative and quantitative analysis of amino acids that composed the proteinaceous polymers found at the interface between calcite crystals deposited by oyster cells and the various metal substrates.

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Title: **Amino Acid Hydrolysis and Analysis System for the Investigation of Site Directed Nucleation and Growth of Ceramic Films on Metallic Surfaces**

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Proposal Narrative

Abstract

An amino acid hydrolysis and analysis system is requested from the Air Force Defense Research Sciences Program. The proposed instrumentation will further the current research effort funded by AFOSR, namely the "Site Directed Nucleation and Growth of Ceramic Films on Metallic Surfaces" by allowing for the qualitative and quantitative analysis of amino acids that compose the proteinaceous polymers found at the interface between calcite crystals deposited by oyster cells and the various metal substrates. A recently described novel biomineralization and deposition process utilizing oyster cells has been manipulated to allow for the formation of a new class of environmentally friendly ceramic coating materials. We are currently in the process of understanding what basic interfacial reactions and chemistries are required for the mechanism of biomineralization and site-specific deposition of ceramic crystals on aluminum alloy substrates. These calcite crystals and resulting ceramic films are deposited onto various metal substrates utilizing the blood cells, or hemocytes, of the Eastern oyster *Crassostrea virginica*. Results to date indicate that site directed crystal deposition by hemocyte cells are regulated by points of cell attachment via particular extra cellular proteins. However, nothing is yet known about the role of these extra cellular molecules in the deposition of the calcite crystals and subsequent formation and growth of a ceramic film. The requested amino acid analysis system consists of integrated modular HPLC ion chromatography components allowing for reconfiguration and upgrades as productivity and research needs demand, a heating/stirring module for the acid hydrolysis of the protein samples, and an evaporator/concentrator for the removal of the acid and concentration of the hydrolysate for analysis on the HPLC. The instrumentation will support data collection on AFOSR contract #F29550-06-1-0133, and allow for the interdisciplinary training of undergraduate and graduate students in the fields of biochemistry and biomaterials into materials science and materials research.

Supporting Information

This request for amino acid hydrolysis and analysis instrumentation is in direct support of a research effort currently funded by the AFOSR Surface and Interfacial Science Program (contract #F29550-06-1-0133, Douglas C. Hansen, Principal Investigator) at the University of Dayton Research Institute (UDRI). The focus of the Surface and Interfacial Science Program is to investigate the relationship between the structure of surfaces and how they react with the surrounding environment at the interface. The Surface Chemistry component of this program is directed at the improved understanding of the mechanisms and phenomena involved in surface processes that will provide for the development of advanced surface structures and interfaces for future Air Force applications.

One area of application is corrosion and materials degradation, with particular interest in the nucleation and growth of thin films and alloys. A recently described novel biomineralization and deposition process utilizing oyster blood cells (hemocytes) from the Eastern oyster *Crassostrea virginica*, could be manipulated to allow for the formation of a new class of environmentally friendly ceramic coating material (1). The understanding of the interfacial reactions necessary for the deposition of a ceramic material at ambient temperature and pressure conditions via a cellular based mechanism is a compelling example of just such a phenomena and surface process.

There are two mineralized layers of shell formed in the Eastern oyster. The first is a thin outlying layer of polycrystalline calcite crystals, known as the prismatic layer. The second is a thick underlying layer of foliated calcite crystals which is called the foliated layer (2). In either case, hemocytes appear to provide crystals for the formation of both layers. There is also a soluble and insoluble organic matrix between the deposited plates that functions as a composite scaffold (3). The objective of the currently funded work is to determine what basic interfacial reactions and chemistries are required for the mechanism of biomineralization and site-specific deposition of these ceramic crystals by hemocytes onto aluminum alloy substrates. The results of the currently funded research effort will lead to an increased understanding of the chemistries involved at oxide-coating interfaces, particularly with regard to ceramic-like films and coatings; the methods used for the deposition of an aqueous crystalline film under ambient conditions may lead to a better utilization of existing methods for the deposition of ceramic coatings that are presently being used. The control of cellular based crystal deposition processes has broad applications to aviation metallurgy and other fields.

The current instrumentation available at UDRI for this research effort will allow the characterization of the interface between the deposited crystals and the various metal substrates currently under study using various microscopy and spectroscopic techniques such as scanning electron microscopy (SEM), laser scanning microscopy (LSM), atomic force microscopy (AFM) and electron backscatter diffraction (EBSD); through collaboration with researchers at the Air Force Research Laboratory (AFRL) Materials Laboratory (ML), similar analytical techniques are available. However, none of these techniques provide chemical information on the composition of the extra cellular matrix proteins that are deposited by the hemocyte in the crystal deposition process or in the formation of the composite scaffold. The capability to quantitatively determine the amino acid composition of these matrix proteins will yield information as to the possible surface binding mechanisms, environmental requirement (pH, surface charge, and surface energy), and putative conformation of these polymers and deposited crystals.

In the past year, we have been able to isolate oyster hemocytes from adult oysters being maintained in our laboratory and incubate them onto untreated glass surfaces and metal-coated glass slides. These cells have then deposited calcite (calcium carbonate) crystals (Figures 1 and 2), thus forming the basis for subsequent deposition of crystals to form the basis of a ceramic coating (Figure 3). These findings represent the first ever reported demonstration of biomineralization by hemocytes isolated from a bivalve on a metal substrate outside of the organism. These results indicate that it is possible to deposit a ceramic material onto a metal substrate at ambient temperature and pressure.

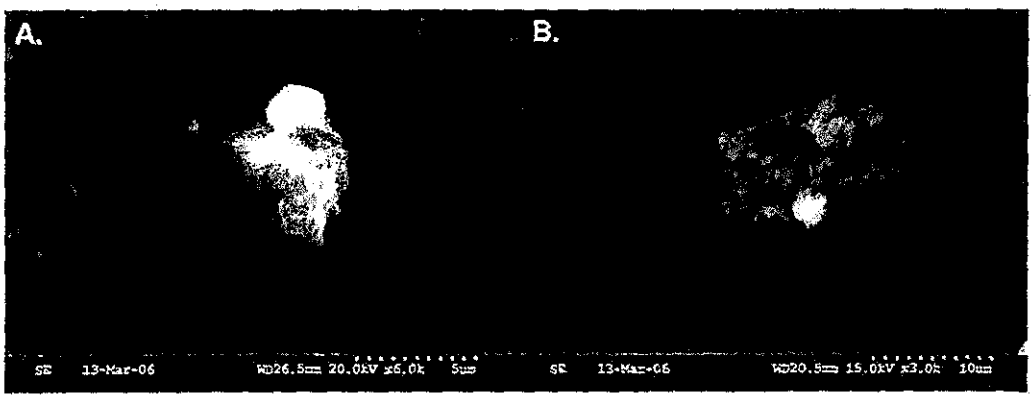

Figure 1 A-B. SEM evidence of cellular biomineralization on smooth glass surfaces. A. Untreated control glass surface has a hemocyte on its surface that has secreted a calcite crystal (white geometric feature). B. Accumulation of crystalline plates onto untreated glass surface. This is a precursor step to a layered formation of ceramic material.

As stated previously, what is not known is the composition and role of extra cellular matrix proteins secreted by the hemocytes in the biomineralization and crystal deposition process. The presence of the extra cellular proteins is visible on the deposited crystals in Figure 3B.

Figure 2 A-C. SEM of promising cell to surface interactions. A. Collagen treated glass surfaces promote cell adhesion of hemocytes. B. 316L Stainless steel polished to a mirror finish promotes cell adhesion and cellular biomineralization. C. Mirror finished Ti6Al4V alloy promotes cell adhesion and biomineralization of crystals.

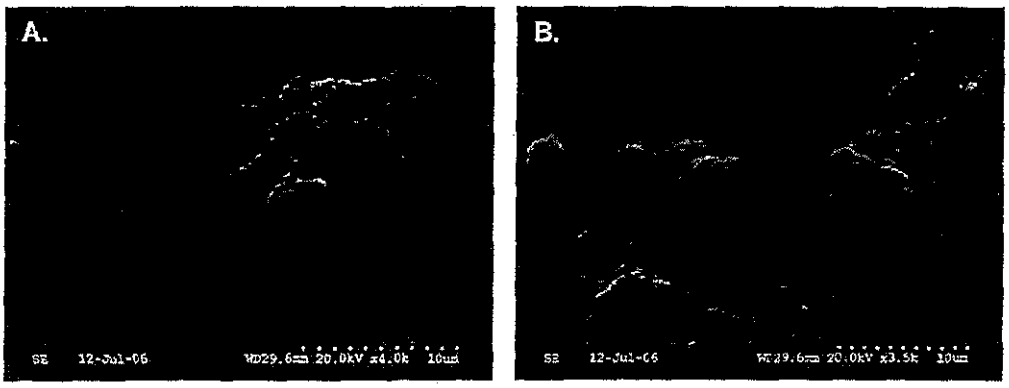

Figure 3 A-B. Evidence of cellular deposition of calcite crystals in layered format. A. Untreated glass slide with an oyster hemocyte attached; note filapodia-like extensions of the hemocyte. B. False color image of cluster of oyster hemocytes and deposited crystals in an ordered array, forming the basis of a deposited ceramic layer.

The interdisciplinary nature of this research combining materials science, biology, biomaterials, and biochemistry requires that the students have the necessary resources to accomplish their research goals. The fact that there is no amino acid analytical capability at the University of Dayton, UDRI or AFRL-ML will significantly impact the progress of the current research effort, both in terms of the training of students in biochemistry, biomaterials and materials science, as well as understanding the mechanism of attachment of the deposited crystals and ceramic biomaterial to the metal substrates. At the present time, 2 undergraduate students and 1 graduate student are being partially supported on this current research effort at UDRI.

Protein samples are routinely analyzed by acid hydrolysis, which break down the protein into its constituent peptides and subsequent amino acids. The AAA-Direct ICS-3000 Ion Chromatography System from Dionex Corporation (Figure 4) separates the amino acids

on a high performance anion-exchange column and directly detects them by integrated pulsed amperometry (Figure 5). This system has advantages over pre- and post-column derivatization methods; pre-column derivatization is highly susceptible to interference from the sample matrix and complex sample matrices can reduce derivatization efficiency, causing high variability in amino acid recovery (4). Post-column derivatization using ninhydrin cannot be performed in samples containing high levels of ammonia due to formation of insoluble complexes that can block instrumentation flow paths (5). The AAA-Direct system can also be used for protein concentration determinations (6).

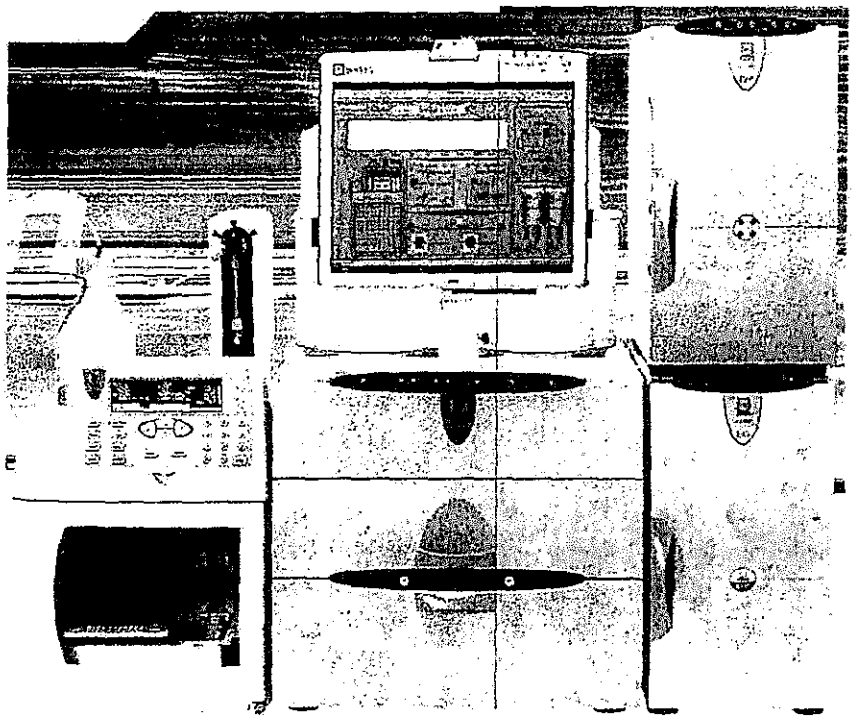

Figure 4. The AAA-Direct ICS-3000 Ion Chromatography System.

The acid hydrolysis of protein samples requires precise heating of the samples in sealed, evacuated ampoules at 110°C for 17 hours. Reacti-Therm II Heating/Stirring Modules are easy to use block heaters that provide constant temperature control (± 0.5° C) and stirring of samples to ensure uniform hydrolysis and dissolution of residues.

After 17 hours, the ampoules are removed from the Reacti-Therm II Heating/Stirring Module and unsealed. The hydrolysate is then transferred to evaporator tubes for the drying and evaporation of the acid in the Thermo DNS SpeedVac Concentrator. This module is designed for consistent drying and evacuation of samples, avoiding the contamination and inconsistent or incomplete removal of acid normally encountered by using conventional rotating water bath evaporators. These two components of the overall

system are critical for reproducible sample preparation, eliminating sources of error in the analysis of amino acids using the AAA-Direct Chromatography instrumentation.

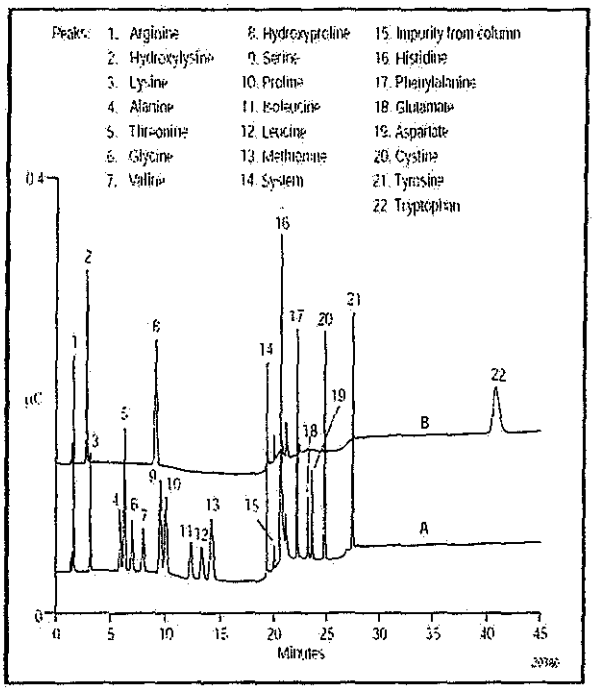

Figure 5. Separation and identification of amino acid standards using AAA-Direct ICS-3000 Ion Chromatography System.

The PI (Hansen) has been provided an office, administrative support, a newly renovated state-of-the-art laboratory of 800 ft^2 with fume hood, 350 liter/hour capacity deionized water system, filtered inert gas (Argon) and air supplies, along with an additional newly renovated laboratory of 425 ft^2 containing a 125 ft^2 Class 10,000 clean room with a laminar flow hood. These facilities are located in the Kettering Engineering Laboratory and Kettering Engineering Laboratory Annex, respectively, on the University of Dayton campus. The Class 10,000 clean room is available for the installation and operation of the requested system; this clean room lab facility minimizes atmospheric contamination of samples and is already being used for dedicated ion chromatography and capillary electrophoresis bench top systems.

The AAA-Direct ICS-3000 system requires a 3 day on site installation and operations training by the manufacturer. No funding for extended warranty or other support program is being requested. The estimated lifetime of all equipment listed in this funding request is 10 years or more.

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